

in corroborating exposure to specific types of fibres in the workplace, the measurement and interpretation of human lung fibre burdens is a complex issue (reviewed by Becklake & Case, 1994; Sébastien, 1994).

Unanswered questions. Given our current knowledge about the properties of fibres relevant for biological activity, the following questions need to be addressed (see Consensus Report):

- To what extent can physico-chemical properties be used to predict the potential carcinogenicity of fibres? Fibre dimensions and durability are currently accepted as important parameters; are there other important characteristics?
- Does the biopersistence of fibres in animals reflect the biopersistence of fibres in humans?
- Is total lung fibre burden an accurate assessment of fibre disposition, or are there localized areas of fibre deposition and retention that correlate with the development of bronchogenic carcinoma and malignant mesothelioma?

Molecular alterations in asbestos-related neoplasms

Malignant mesothelioma

Malignant neoplasms are characterized by autonomous proliferation and multiple alterations in oncogenes and tumour suppressor genes (reviewed by Harris, 1992). Exposure to asbestos fibres may contribute directly or indirectly to these molecular alterations, as hypothesized by Walker *et al.* (1992). Alterations in growth regulatory genes, oncogenes and tumour suppressor genes have been investigated in human and rodent mesothelial cell lines, although few investigations have been carried out directly on primary tumours. Multiple alterations in oncogenes and tumour suppressor genes have also been described in various histological types of human bronchogenic carcinomas (reviewed by Viallet & Minna, 1990); however, few investigations have been carried out in patients who developed bronchogenic carcinoma following asbestos exposure alone or in combination with cigarette smoking. Three categories of molecular alterations have been investigated in human malignant mesothelioma cell lines: (i) alterations in oncogenes and growth factors; (ii) alterations in

growth regulatory genes; and (iii) alterations in tumour suppressor genes. These experimental data will be summarized briefly.

Oncogenes and growth factors. Several investigators have examined human malignant mesothelioma cell lines for the activation of common oncogenes by point mutation or amplification: no changes have been found yet in the *ras* gene family. A novel oncogene has been discovered in a human mesothelioma cell line using an NIH 3T3 transfection assay; however, its sequence has not yet been determined (Walker *et al.*, 1992). Some oncogenes encode for growth factors or their receptors; activation of these oncogenes up-regulates cell proliferation in neoplastic cells. The *c-sis* proto-oncogene that encodes for the B chain of platelet-derived growth factor (PDGF-B) is expressed in malignant, but not normal, human mesothelial cell lines. However, over-expression of PDGF-A chain appears to be more important for the autonomous proliferation of human mesothelial cells as determined by transfection studies using an immortalized mesothelial cell line (Van der Meeren *et al.*, 1993). A second autocrine growth factor loop involving transforming growth factor- α (TGF- α) has also been reported in human mesothelioma cell lines (Morocz *et al.*, 1994). No evidence for the amplification of the genes encoding for the epidermal growth factor receptor or the *met* growth factor receptor was found in human malignant mesotheliomas (Tiainen *et al.*, 1992).

Growth regulatory genes. Cell proliferation in mammalian cells is controlled by the expression of cyclins and cyclin-dependent kinases, which are turned on and off in a precise temporal sequence during the cell cycle. Over-expression of a cyclin would result in continuous stimulation of the cell cycle and increased cell proliferation. Cyclin D1 is an important regulator of the G1 phase of the cell cycle; over-expression of the cyclin D1 protein has been reported in a series of human cancer cell lines, including two malignant mesothelioma cell lines (Schauer *et al.*, 1994).

Cyclins, as well as cyclin-dependent kinases, function to drive cell cycle progression. Cyclin-dependent kinase inhibitors function as brakes, opposing the action of kinases. An important

growth inhibitor is cyclin-dependent kinase 4 inhibitor (p16). This growth inhibitor has been found to be mutated or deleted in the majority of human carcinoma cell lines, including malignant mesotheliomas (Gerwin, 1994). These recent investigations provide evidence for the autocrine activation of at least two growth factor pathways (PDGF and TGF- α), in addition to the inactivation of p16, an important regulator of the G1 phase of the cell cycle. Together, these alterations could contribute to the autonomous or unregulated proliferation of malignant mesothelioma cells.

Tumour suppressor genes. Three tumour suppressor genes have been investigated in human malignant mesotheliomas: *Rb* (the retinoblastoma gene), *WT1* (the Wilm's tumour gene) and *p53* (the most commonly altered tumour suppressor gene, mutated in approximately 50% of human neoplasms). No alterations have been found in *Rb* in human mesothelioma cell lines; this finding is not surprising because over-expression of cyclin D1 may substitute for deletion of *Rb*. *WT1* is expressed in both normal and malignant human mesothelial cell lines; so far, no deletions of this tumour suppressor gene have been found in human malignant mesotheliomas, although more subtle alterations in this gene may be responsible for altered growth factor responses in these neoplasms (Gerwin, 1994). Two groups of investigators have examined human malignant mesothelioma cell lines for alterations in the tumour suppressor gene *p53*. Overall, only one-third of the cell lines investigated showed any alterations. In contrast to most human bronchogenic carcinomas, point mutations in the highly conserved exons 5–8 of this gene were rare (Cote *et al.*, 1991; Metcalf *et al.*, 1992).

The infrequent association of point mutations in *p53* with human malignant mesothelioma conflicts with the results of immunohistochemical assays to detect expression of *p53* protein in human mesotheliomas *in situ*. Between 25 and 45% of these human neoplasms show increased expression of *p53* (Mayall *et al.*, 1992; Ramael *et al.*, 1992). Increased *p53* expression is usually associated with point mutations (in *p53*) that prolong the half-life of this nuclear phosphoprotein. In the absence of point mutations, other mechanisms must be responsible for the elevated level of *p53* expression in human malignant mesotheliomas.

Bronchogenic carcinoma

The genetic lesions associated with the activation of oncogenes or inactivation of tumour suppressor genes may be indicative of exposure to specific chemical carcinogens (Hollstein *et al.*, 1991). For example, guanine → thymine transversions are induced by carcinogens in cigarette smoke; this mutation occurs frequently at codon 12 of the *K-ras* proto-oncogene in adenocarcinomas of the lung arising in smokers and ex-smokers (Westra *et al.*, 1993). The same mutation at this locus has also been shown to occur in lung adenocarcinomas of workers exposed to both asbestos and cigarette smoke (Husgafvel-Pursiainen *et al.*, 1993). Specific carcinogens in cigarette smoke most probably initiate this mutation in *K-ras* at an early stage in the development of lung adenocarcinomas (Westra *et al.*, 1993).

Accumulation of *p53* has also been detected in bronchogenic carcinomas arising in patients exposed to asbestos and cigarette smoke. Also, as demonstrated by immunohistochemistry, *p53* is over-expressed in lung carcinomas associated with cigarette smoking alone or in combination with asbestos exposure (Nuorva *et al.*, 1994). So far, the pattern of *p53* mutations in bronchogenic carcinomas associated with asbestos exposure alone or in combination with cigarette smoke has not been analysed. In cigarette smokers, over-expression of *p53* resulting from point mutations in exons 5–8 is found in pre-neoplastic and dysplastic lesions adjacent to invasive neoplasms; thus, mutations in this tumour suppressor gene are also considered to be an early event in the development of bronchogenic carcinoma (Cagle, 1995).

Additional comparative studies between cell lines and archival tissue samples of experimental and human lung tumours associated with asbestos exposure are required to evaluate the role of mineral fibres in the induction of molecular alterations during the development of bronchogenic carcinoma and malignant mesothelioma.

Mechanisms of fibre carcinogenesis

Some clues about the mechanisms responsible for asbestos-induced carcinogenesis have been provided by (i) the physico-chemical properties of fibres known to be carcinogenic for humans and (ii) recent cellular and molecular investigations of human malignant mesothelioma and bronchogenic carcinoma. Although comparative studies of the

effects of asbestos and other natural and man-made fibres in *in-vitro* and *in-vivo* models are incomplete, the following five hypotheses are proposed.

Hypothesis 1: Fibres generate free radicals that damage DNA

Natural and man-made fibres have been shown to catalyse the generation of ROS in cell-free or *in-vitro* model systems (reviewed by Kamp *et al.*, 1992), and these ROS may indirectly cause genetic damage. The following mechanisms are proposed for generation of oxidants. First, fibres may directly participate in the iron-catalysed generation of ROS in the presence of molecular oxygen, superoxide anion or hydrogen peroxide. The hydroxyl radical (OH^{\bullet}) is the end-product of these reactions and it has been shown to modify DNA bases in a cell-free system (Leanderson *et al.*, 1988) and an *in-vitro* cell model system (Takeuchi & Morimoto, 1994). Asbestos fibres have also been shown to catalyse iron-dependent oxidation of membrane lipids (Gulumian & Kilroe-Smith, 1987; Goodlick *et al.*, 1989) producing reactive alkoxyl radicals (Kamp *et al.*, 1992). Second, independently of iron or oxygen, asbestos fibres can also catalyse oxidation of PAH to a free radical (Graceffa & Weitzman, 1987). Third, fibres may activate phagocytes to release ROS. It is hypothesized that 'frustrated phagocytosis' of long fibres is a potent stimulus for release of ROS (Mossman & Marsh, 1989). After release of hydrogen peroxide, superoxide anion or nitric oxide from activated phagocytes, additional radicals may be generated including OH^{\bullet} , peroxynitrite and nitronium ions (Ohshima & Bartsch, 1994). It is hypothesized that chronic generation of these various types of free radicals at sites of fibre deposition or during a persistent chronic inflammatory reaction mediates DNA damage that leads to critical mutations in oncogenes, growth regulatory genes and tumour suppressor genes. The evidence for and against this hypothesis that fibres generate free radicals that damage DNA will be summarized briefly below.

Assays for DNA damage. Most of the experimental studies searching for evidence of DNA damage induced by fibres have been conducted with various target cell populations *in vitro*. As reviewed by Jaurand (1989, 1991), the evidence for DNA damage is conflicting depending on the following variables: (i) fibre source and preparation, (ii) cell

type, (iii) species and (iv) assay conditions. In general, asbestos fibres are considered to be inactive or weak inducers of point mutations or sister chromatid exchange. Direct measurement of DNA strand breaks has also been unsuccessful. However, asbestos fibres have been shown to induce multilocus deletions in a human-hamster hybrid cell line (Hei *et al.*, 1992) and a variety of natural and man-made fibres have been shown to be clastogenic for rodent cells (Dong *et al.*, 1994), but less damaging to human cells (Kinnula *et al.*, 1995). Mesothelial cells appear to be more sensitive to DNA damage induced by asbestos fibres than do epithelial cells or fibroblasts; this increased sensitivity may be related to different levels of antioxidant defence mechanisms (Kinnula *et al.*, 1992).

Cellular responses to DNA damage. Although direct measurements of DNA damage induced by fibres have been inconsistent, several indirect effects of DNA damage have been measured. For example, asbestos fibres have been shown to induce DNA repair synthesis (reviewed by Jaurand, 1991) and genetic responses to oxidant stress (Janssen *et al.*, 1994). Tissue and species differences in these adaptation and repair pathways may account for discrepancies among different *in-vitro* model systems. These differences may also be apparent in human populations exposed to fibres.

Individual susceptibility to oxidant stress and altered DNA repair mechanisms. Individual adaptive responses to oxidant stress and the ability to repair damaged DNA are dependent on multiple exogenous and endogenous factors. Few experiments have attempted to manipulate these variables in animal or human model systems to test the hypothesis that free radicals play a role in fibre carcinogenesis. Only the following two studies using human cells *in vitro* have investigated genetic susceptibility to injury induced by asbestos fibres: fibroblasts from patients with a defect in DNA repair were found to be more sensitive to asbestos toxicity (Yang *et al.*, 1984); and primary mesothelial cells obtained from individuals lacking glutathione S-transferase (GST) M1 were also more sensitive to asbestos toxicity (Pelin *et al.*, 1995).

As described earlier, asbestos fibres have been shown to catalyse lipid peroxidation, generating

organic hydroperoxides and other lipid-derived radicals that may damage DNA. One defence mechanism against organic hydroperoxides is reduction by GST. Approximately 50% of people carry a homozygous deletion of the GSTM1 isoenzyme. Pelin *et al.* (1995) explored the hypothesis that the *GSTM1* null genotype predisposes to asbestos-induced genetic damage and the development of malignant mesothelioma. No increase in the frequency of the *GSTM1* null genotype was found in a series of 44 patients with asbestos-related malignant mesothelioma. Mesothelial cell lines derived from people with the *GSTM1* null genotype showed increased toxicity in response to asbestos, but no increase in cytogenetic abnormalities. This study suggests that alterations in GSTM1 activity do not predispose to cytogenetic damage or malignant mesothelioma associated with asbestos exposure.

Rare inherited chromosomal instability syndromes exist that reflect abnormalities in various DNA repair pathways. Patients with xeroderma pigmentosum, ataxia telangiectasia or Bloom's syndrome have increased susceptibility to cancer induced by ultraviolet or ionizing radiation. Only one study has investigated the sensitivity of fibroblasts derived from patients with xeroderma pigmentosum to asbestos fibres *in vitro*. This study reported increased cytotoxicity in these cultures (Yang *et al.*, 1984). Additional experiments to confirm and expand this finding are required.

Hypothesis 2: Fibres interfere physically with mitosis

An important characteristic of natural and man-made fibres is their ability to induce aneuploidy, polyploidy and binucleate cells in a variety of cells cultured *in vitro* (reviewed by Jaurand, 1989, 1991). In contrast to the clastogenic effects of fibres, the aneuploidogenic effects of fibres can be demonstrated in a wide variety of cell types (with the exception of lymphocytes) derived from both rodents and humans. On the basis of these observations, it is hypothesized that the aneuploidogenic effects of fibres contribute to their carcinogenicity. The experimental observations that support this hypothesis will be discussed briefly.

Requirement for phagocytosis. For fibres to interfere physically with the mitotic apparatus, two requirements must be fulfilled. First, fibres must

be internalized by the target cell population. With the exception of lymphocytes, most types of cells phagocytize fibres *in vitro*. This process may be facilitated by binding to specific membrane receptors; for example, crocidolite asbestos binds to the scavenger receptor of macrophages (Resnick *et al.*, 1993). Second, the target cell population must be proliferating; this requirement will be discussed in detail subsequently. While both of these requirements are achieved readily *in vitro*, it has not been established whether asbestos fibres are internalized by the target cells giving rise to bronchogenic carcinoma or malignant mesothelioma *in vivo*. Few measurements of asbestos fibre burdens have been made on pleural tissue; in two studies, predominantly short fibres were found, which did not correlate with the lung fibre burden (Sébastien *et al.*, 1980; Dodson *et al.*, 1990). Additional studies to localize fibres in the target cell populations of the pleura and bronchial epithelium are required to test this hypothesis *in vivo*.

Fate of internalized fibres. Previous morphological studies of cells exposed to asbestos fibres *in vitro*, then examined by light or electron microscopy, demonstrated direct physical interaction between fibres and the nucleus or chromosomes during mitosis (Wang *et al.*, 1987; Jaurand, 1991). More recent investigations demonstrate that internalized fibres are surrounded by a phagolysosomal membrane thereby preventing direct physical or chemical interaction with or adsorption to chromosomes (Jensen *et al.*, 1994). Both short and long crocidolite asbestos fibres were observed to accumulate in the perinuclear region of amphibian lung epithelial cells *in vitro*; short fibres showed saltatory transport along microtubules while long fibres remained stationary (Cole *et al.*, 1991). Approximately 10–20 fibres were internalized by these large epithelial cells *in vitro* without any adverse effects on viability or cell division (Ault *et al.*, 1995).

Mechanisms of interference with mitosis. Previous studies using light microscopy on fixed cells suggested physical interference of long fibres with the mitotic spindle and chromosomal segregation, which resulted in lagging or sticky chromosomes during anaphase and the subsequent generation of aneuploid daughter cells (Hesterberg & Barrett, 1985). A recent study using video-enhanced

time-lapse microscopy of living amphibian lung epithelial cells during mitosis has extended these initial observations (Ault *et al.*, 1995). In this cell type, a keratin microfilament cage surrounds the nucleus, and this architecture is maintained during mitosis. Long crocidolite asbestos fibres were observed to be trapped in this keratin microfilament cage protruding into the spindle region of mitotic cells. In most cases, collisions between the protruding asbestos fibre and chromosomes did not interfere with their orderly segregation; there was no evidence for sticking of chromosomes to fibres. In rare cases, a protruding fibre did impede chromosomal migration to the spindle pole or cause a chromosomal break. These investigators hypothesize that long fibres are more easily trapped in the keratin microfilament cage than short fibres and that they are more likely to protrude into the spindle region during mitosis. It is proposed that the keratin cage is disrupted during mitosis of mesothelial cells, allowing long fibres to interfere more readily with mitosis in this cell type (Ault *et al.*, 1995).

In addition to physical interference with chromosomal segregation, fibres may also disrupt mitosis by other mechanisms. Exposure of human mesothelial cells to amosite fibres *in vitro* caused disruption of their cytoskeletal organization; this disruption could enhance the ability of fibres to interfere with the mitotic spindle (Somers *et al.*, 1991). In addition to changes in chromosomal number, asbestos fibres also induce binucleate daughter cells in dividing cell populations *in vitro*. This effect is postulated to be the result of physical interference of fibres with the cleavage furrow (Kenne *et al.*, 1986).

Are these mechanisms responsible for the cytogenetic alterations observed in experimental and human mesothelioma cell lines? Multiple non-random cytogenetic alterations are characteristic of experimental and human mesothelioma cell lines (reviewed by Barrett, 1994). Monosomy, polysomy and partial deletions are frequent, especially in human chromosomes 1, 3 and 5-9. The relationship between chromosomal alterations induced by fibres and carcinogenesis is supported by the cytogenetic alterations that accompany *in-vitro* transformation of SHE cells or rat pleural mesothelial cells exposed to fibres

(reviewed by Walker *et al.*, 1992). However, not all cell types are transformed by exposure to asbestos fibres *in vitro*, although they do show aneuploidy (reviewed by Jaurand, 1991). Additional mechanisms may contribute to the genetic alterations responsible for the development of bronchogenic carcinomas and malignant mesotheliomas associated with exposure to asbestos fibres. First, oxidative damage to DNA has recently been shown to alter the methylation pattern of adjacent cytosines. Altered gene methylation, as well as point mutations resulting from mispairing at sites of oxidized bases, may alter gene expression in malignant neoplasms (Weitzman *et al.*, 1994). Second, the cytogenetic abnormalities characteristic of malignant mesotheliomas may result from genetic instability arising from loss of the G1 cell cycle checkpoint (Lane, 1992). Inactivation of normal p53 disrupts this G1 cell cycle checkpoint. Altered p53 function can result from deletion or point mutation of the p53 tumour suppressor gene or binding to viral proteins such as the SV40 T-antigen. As summarized earlier, mutations in the p53 tumour suppressor gene occur frequently in human bronchogenic carcinomas, although the pattern of p53 mutations in patients who were also exposed to asbestos has not been investigated. Point mutations in p53 are infrequent in human malignant mesotheliomas; in addition, no mutations in MDM2, which codes for a p53-binding protein, were found in human mesothelioma cell lines (Gerwin, 1994). However, over-expression of p53 has been demonstrated in up to one-half of human malignant mesotheliomas (Mayall *et al.*, 1992; Ramael *et al.*, 1992). Although the functional status of the G1 cell cycle checkpoint has not been studied in human mesothelial cell lines, inactivation of the G1 cell cycle checkpoint could be responsible for genetic instability and multiple chromosomal alterations observed in human malignant mesotheliomas (Moyer *et al.*, 1994).

Unanswered questions. At least two mechanisms responsible for the genotoxic effects of fibres have been proposed: (i) indirect DNA damage mediated by free radicals; and (ii) direct physical interference with the mitotic apparatus. These mechanisms are supported by observations of the genotoxic effects of asbestos fibres using cell cultures *in vitro*. The following questions must be

addressed in order to validate these proposed mechanisms:

- Are *in-vitro* genotoxicity assays relevant to fibre carcinogenesis?
- Are *in-vitro* doses relevant for *in-vivo* exposures?
- Can the genotoxic effects of fibres be assessed *in vivo*?

Hypothesis 3: Fibres stimulate the proliferation of target cells

Cell proliferation is required for the fixation of mutations induced by genotoxic agents. Clonal expansion of initiated or pre-neoplastic cell populations is accelerated in a growth-stimulatory environment. An increased rate of cell proliferation also leads to populations of cells with increased levels of spontaneous mutations (reviewed by Barrett, 1994). Cell proliferation is balanced by terminal differentiation and death of cells by apoptosis. As summarized below, exposure to asbestos fibres under certain conditions is accompanied by cell proliferation; the effects of fibres on terminal differentiation and apoptosis have not yet been explored.

Experimental observations *in vitro* and *in vivo*. At high levels of exposure *in vitro*, asbestos fibres are toxic to a variety of target cell populations. This toxicity is hypothesized to be mediated by the generation of ROS (reviewed by Mossman & Marsh, 1989) or the inhibition of cell proliferation by physical interference with mitosis (Hesterberg & Barrett, 1985). However, under some conditions *in vitro*, asbestos fibres induce proliferation of hamster tracheobronchial epithelial cells (Sesko *et al.*, 1990), rat lung fibroblasts (Lasky *et al.*, 1995) and rat pleural mesothelial cells (Heintz *et al.*, 1993). Cell proliferation in these same target cell populations in the lung and pleura has also been observed following intratracheal instillation (Dodson & Ford, 1985; Adamson *et al.*, 1993), inhalation (Brody *et al.*, 1989) or intraperitoneal injection of asbestos fibres (Moalli *et al.*, 1987; Friemann *et al.*, 1990). These early proliferative responses occur in cell populations where a malignant neoplasm may arise, that is, the bronchial epithelium, type II alveolar epithelial cells and mesothelial cells. However, proliferation of macrophages, interstitial fibroblasts and endothelial cells is also observed in these *in-vivo* models

(Branchaud *et al.*, 1989; Brody *et al.*, 1989); these early proliferative reactions are associated with angiogenesis and fibrosis characteristic of a wound-healing response.

Pott (1979) has proposed a mechanistic hypothesis linking the biopersistence of fibres at the target tissue with the stimulation of cell proliferation; this hypothesis states that fibres must persist and stimulate a sufficient number of population doublings to give rise to a pre-neoplastic cell population (Pott, 1987). So far, this hypothesis has not been tested experimentally.

Mechanisms responsible for stimulation of cell proliferation. Experimental evidence has been obtained for four potential mechanisms of growth stimulation in response to asbestos fibres: (i) compensatory cell proliferation in response to toxicity; (ii) stimulation of intracellular signal transduction pathways; (iii) direct mitogenesis; and (iv) induction of growth factor and growth factor receptor expression.

The evidence for these mechanisms will be summarized briefly. These potential mechanisms require direct interaction with or phagocytosis of fibres by the target cells. Alternatively, an indirect mechanism leading to the stimulation of cell proliferation is through the release of cytokines and growth factors from inflammatory cells. This indirect mechanism does not require direct contact between fibres and the target cell population; this indirect pathway will be discussed subsequently.

Injury to target cells has been demonstrated in the parietal mesothelial lining after direct intraperitoneal injection of crocidolite asbestos fibres in mice (Moalli *et al.*, 1987). Localized damage to the alveolar epithelium has also been proposed to facilitate translocation of fibres and growth factors into the interstitium of the lungs following either inhalation (Brody *et al.*, 1989) or intratracheal instillation of asbestos fibres (Adamson & Bowden, 1988). Cell proliferation is triggered at these localized sites of injury induced by the deposition of asbestos fibres. In the parietal mesothelium, the morphology and kinetics of mesothelial cell proliferation resemble the healing response of this tissue as triggered by other types of injury (Moalli *et al.*, 1987).

A second mechanism leading to stimulation of cell proliferation by fibres is the triggering of

intracellular signal transduction pathways. Several of these biochemical events are common to asbestos fibres and other tumour promoters such as phorbol esters. There is substantial experimental evidence for the action of asbestos fibres as a tumour promoter, especially for the tracheo-bronchial epithelium (Hoskins *et al.*, 1991). For example, using the model of hamster tracheal epithelial cells exposed to fibres *in vitro*, B.T. Mossman and co-workers demonstrated the following effects of asbestos fibres: increased expression of ornithine decarboxylase (Marsh & Mossman, 1991); activation of protein kinase C (Perderiset *et al.*, 1991); and hydrolysis of inositol phospholipids (Sesko *et al.*, 1990). Some of these biochemical changes may be initiated by ROS (Marsh & Mossman, 1991).

A third mechanism leading to cell proliferation is direct mitogenesis in the absence of cell toxicity. Direct mitogenesis requires phagocytosis or the binding of the fibres to cell surface receptors, although this latter mechanism has not been explored in cells other than phagocytes. B.T. Mossman and co-workers have obtained evidence for the mitogenic effects of fibres *in vitro* based on induction of proto-oncogene expression. In contrast to soluble tumour promoters such as phorbol esters, asbestos fibres cause a prolonged expression of the proto-oncogenes *c-fos* and *c-jun*, which may be responsible for the persistent growth stimulation of target cells (Heintz *et al.*, 1993).

Finally, asbestos fibres may also trigger cell proliferation by inducing the expression of growth factors and growth factor receptors, activating an autocrine growth-stimulatory pathway. Growth activation by this mechanism has been demonstrated by increased expression of PDGF-AA and its receptor by rat lung fibroblasts exposed to chrysotile asbestos *in vitro* (Lasky *et al.*, 1995). The missing link between exposure to fibres and induction of gene expression is the identification of the mechanism responsible for the turning on of transcription factors that regulate specific genes. One potential mechanism is oxidant stress induced by generation of ROS leading to activation of the nuclear transcription factor kB (NF- κ B) (Moyer *et al.*, 1994).

Unanswered questions. Exploration of the effects of fibres on gene expression and cell proliferation

is an emerging area of research; experiments must be designed to address the following question:

- What is the relationship between (i) the acute effects of fibres on gene expression and (ii) chronic, persistent proliferation of target cell populations?

Hypothesis 4: Fibres provoke a chronic inflammatory reaction leading to the prolonged release of ROS, cytokines and growth factors in the lungs

Macrophages are the initial target cells of fibres and other particulates that deposit in the lungs or pleural and peritoneal spaces. Phagocytosis of asbestos fibres is accompanied by the activation of macrophages, which results in the increased synthesis and secretion of ROS as well as a variety of chemical mediators and cytokines. These mediators amplify the local inflammatory reaction. Persistence of asbestos fibres in the interstitium of the lungs or in the subpleural connective tissue may lead to a sustained chronic inflammatory reaction accompanied by fibrosis (reviewed by Driscoll, 1993; Oberdörster, 1994). The mechanisms responsible for these inflammatory reactions will be reviewed briefly.

Recruitment of inflammatory cells. Migration of macrophages to sites of asbestos fibre deposition is mediated by activation of chemotactic factors. In the lungs, the chemotactic factor, C5a, has been shown to play an important role in the recruitment of macrophages (reviewed by Rom *et al.*, 1991). Activated macrophages also synthesize arachidonic acid metabolites such as leukotriene B4, which are chemotactic for neutrophils. Activated macrophages also express cytokines, including interleukin-1 and tumour necrosis factor- α . These cytokines sustain and amplify the initial inflammatory response by triggering the release of additional chemotactic factors called chemokines (interleukin-8 and macrophage inflammatory protein 2) from macrophages, pulmonary epithelial cells (Driscoll, 1993) and mesothelial cells (Griffith *et al.*, 1994). **Mediators released from inflammatory cells.** Direct stimulation by phagocytosis of fibres (Donaldson *et al.*, 1989, 1992) and indirect stimulation by cytokines triggers the synthesis and release of additional mediators. Neutrophils and macrophages release ROS as described above; these

may cause DNA and chromosomal damage. In addition, proteolytic and hydrolytic enzymes may be released, which damage basement membranes and connective tissue in the lungs. Growth factors for epithelial cells and fibroblasts, such as TGF- α and PDGF, are also released from activated macrophages. Release of these mediators is a nonspecific response to lung injury and inflammation; overall, there is a balance between these cytokines and growth factors, which restores the lung to its original function and structure. It is hypothesized that an imbalance between these cytokines and growth factors may contribute to the pathological effects of asbestos fibres, especially diffuse interstitial pulmonary fibrosis or asbestosis (Oberdörster, 1994).

Biological effects of inflammatory mediators.

Unregulated or persistent release of these inflammatory mediators may lead to tissue injury, scarring by fibrosis and proliferation of epithelial and mesenchymal cells. The cytotoxic effects of asbestos fibres are amplified in the presence of neutrophils in *in-vitro* models (Kamp *et al.*, 1989; Kinnula *et al.*, 1995). Damage to the alveolar epithelial lining and basement membrane is especially dangerous because asbestos fibres and inflammatory mediators gain access to the interstitium of the lung (Rom *et al.*, 1991). The interrelationship between inflammation, lung injury and fibrosis has been established in two animal models. First, exposure of complement-deficient mice to chrysotile asbestos fibres resulted in reduced levels of macrophage accumulation in sites of fibre deposition and this attenuated the subsequent fibrotic reaction (McGavran *et al.*, 1989). Second, lung inflammation and fibrosis induced by inhalation of crocidolite asbestos fibres was decreased by polyethylene glycol conjugated to catalase, implicating ROS in lung injury produced by fibres (Mossman *et al.*, 1990).

Pulmonary fibrosis is frequently accompanied by proliferation of type II alveolar epithelial cells (Kuhn *et al.*, 1989). The relationship between sustained epithelial cell proliferation, diffuse interstitial pulmonary fibrosis and bronchogenic carcinoma is controversial.

Mesothelial cell proliferation, especially involving the visceral pleura, is also an early reaction to intratracheal instillation of asbestos fibres. However, at these early time points, no fibres have been

observed in the visceral pleura and this proliferative response is hypothesized to be mediated indirectly by cytokines and growth factors released from activated interstitial macrophages (Adamson *et al.*, 1994). The relationship between chronic or persistent release of cytokines and growth factors, chronic mesothelial cell proliferation and the development of malignant mesothelioma remains to be tested.

Unanswered questions. In the lungs and pleural linings, chronic inflammation and fibrosis are common reactions following exposure to asbestos fibres. An important mechanistic question remains to be answered:

- What are the links between inflammation, fibrosis and cancer induced by fibres?

Hypothesis 5: Fibres act as co-carcinogens or carriers of chemical carcinogens to the target tissue Cigarette smoke, asbestos and lung cancer. Epidemiological studies have confirmed that cigarette smoke plus asbestos fibres are multiplicative in the induction of bronchogenic carcinoma (Selikoff *et al.*, 1968; Saracci, 1977). Experiments using cell culture and animal models provide evidence that asbestos fibres enhance the delivery of multiple carcinogens in cigarette smoke to the bronchial epithelium and increase their metabolic activation. For example, cigarette smoking retards ciliary action and clearance of fibres and other particulates (McFadden *et al.*, 1986). Also, ROS in cigarette smoke or generated by iron-catalysed reactions in the presence of asbestos fibres enhance fibre uptake by tracheal epithelial cells (Hobson *et al.*, 1990). Both crocidolite and chrysotile asbestos also enhance cellular uptake and metabolic activation of benzo[a]pyrene in hamster tracheal epithelial cells (Mossman *et al.*, 1983). On the basis of these experimental observations, it is hypothesized that asbestos fibres and cigarette smoke act as co-carcinogens as summarized in Fig. 1.

As reviewed earlier, asbestos fibres may also contribute by additional mechanisms to the development of bronchogenic carcinoma, either alone or in combination with cigarette smoke. For example, asbestos and cigarette smoke act synergistically to damage DNA in a cell-free system; this

DNA damage has been shown to be mediated by the iron-catalysed generation of OH[•] (Jackson *et al.*, 1987). In hamster tracheobronchial rings maintained in organ culture, asbestos fibres induce squamous metaplasia, a potentially reversible precursor lesion that may evolve into bronchogenic carcinoma (Mossman *et al.*, 1977). Finally, asbestos fibres have been shown to stimulate the proliferation of tracheal epithelial cells and to mimic the biochemical effects of tumour promoters as discussed above.

Interactions between fibrous and non-fibrous dusts. People are commonly exposed to mixtures of fibrous and non-fibrous dusts, even in the ambient environment. Man-made fibres are also mixtures of fibrous and non-fibrous materials. The toxicity of particulates containing quartz is well recognized; however, less is known about the potential additive or synergistic effects between fibrous and less toxic non-fibrous dusts. Exposure of rodents to mixed dusts by inhalation resulted in increased transport of fibres across the visceral pleura and increased production of lung tumours and mesothelioma (reviewed by Oberdörster, 1994).

Unanswered questions. These experiments in rodents raise the following concern about exposure of humans to mixed fibrous and non-fibrous dusts:

- Does inhalation of fibres or mixed fibrous and non-fibrous dusts impair clearance in rats? Is this mechanism relevant for humans?

Caveats about fibres and carcinogenesis

The five mechanistic hypotheses above have been proposed for the role of asbestos fibres in the development of bronchogenic carcinoma and malignant mesothelioma. As summarized above and in the accompanying reviews, there is experimental evidence to support each of these mechanisms; however, there is not yet conclusive evidence for or against any of these mechanisms. Most of the current experimental data are derived from observations with asbestos fibres and a few types of man-made fibres. For these reasons, the following caveats must be considered:

Different types of fibres may act by different mechanisms. Experimental evidence has been presented for genetic and epigenetic roles of asbestos fibres in carcinogenesis. Different fibre types may show predominantly genetic or epigenetic effects. ROS are hypothesized to mediate some of the genetic and epigenetic effects of asbestos fibres. This mechanism must be evaluated critically for each type of natural and man-made fibre tested in *in-vitro* or *in-vivo* models. The problem of acute versus chronic or persistent effects must be addressed in appropriate animal model systems.

Different mechanisms may contribute to the development of bronchogenic carcinoma and malignant mesothelioma. The interaction between asbestos exposure and cigarette smoking in the development of bronchogenic carcinoma has

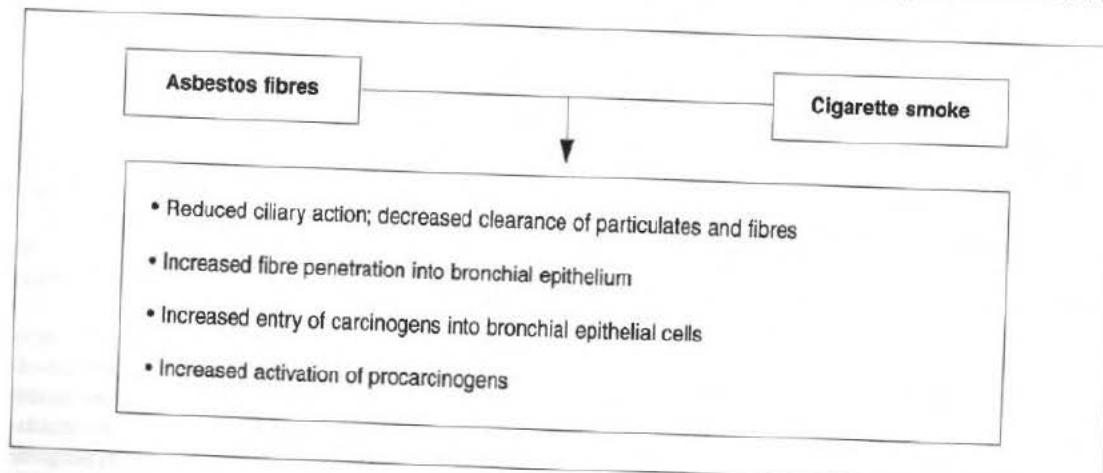


Figure 1. Hypothetical co-carcinogenic action of asbestos fibres and cigarette smoke.

been well documented. The experimental and epidemiological data support a role for asbestos fibres during the promotion stage of the development of bronchogenic carcinoma. Mechanisms have been proposed for the action of asbestos fibres as both initiators and promoters in the development of malignant mesothelioma.

Other potential mechanisms. The pathogenesis of malignant mesothelioma has not yet been established; therefore, it is important to explore all potential mechanisms. For example, Appel *et al.* (1988) demonstrated that asbestos fibres can transfet plasmid DNA into cells *in vitro*. Transfection of exogenous DNA can be mediated by other agents, including calcium phosphate precipitates, so the specificity of this mechanism for asbestos fibres was questioned. Recently, SV40-like DNA sequences were identified in 60% of human mesothelioma tissue samples, but not in adjacent lung tissue or in 47 other tumour samples studied. The origin of this viral DNA and the specificity for malignant mesothelioma tissue is unknown (Carbone *et al.*, 1994). Between 1954 and 1961, people ingested live oral polio vaccine that may have been contaminated with SV40 virus. Many people are exposed to related papovaviruses such as JC virus in childhood; this virus becomes latent and persists in the kidney. A related papovavirus, BK, has been found in brain tumours, Kaposi's sarcoma and in pancreatic islet-cell tumours (King, 1993). Asbestos fibres may transfet viral DNA into mesothelial cells. The presence of SV40-related DNA sequences and expression of the viral T-antigen would explain the high percentage of p53 immunoreactivity in human malignant mesothelioma tissue as discussed above (see *Molecular alterations in asbestos-related neoplasms*).

Alternatively, reactivation of a latent papovavirus infection in patients with malignant mesothelioma may be associated with an immunocompromised state. Alterations in cell-mediated immunity and natural killer cell activity have been described in patients with asbestosis (reviewed by Rom *et al.*, 1991). It is unknown whether altered immunosurveillance contributes to the development of asbestos-related neoplasms.

Fibres may contribute to carcinogenesis by multiple mechanisms. As discussed by Barrett (1994), fibres may act at multiple stages in neo-

plastic development. The co-carcinogenic effects of asbestos fibres and cigarette smoke in metabolic activation of chemical carcinogens have been summarized in Fig. 1. On the basis of molecular studies of lung cancers associated with cigarette smoking and asbestos exposure, it is proposed that the initiation of mutations in the early stages of the development of bronchogenic carcinomas reflects the mutagenic activity of agents found in cigarette smoke (Husgafvel-Pursiainen *et al.*, 1993; Nuorva *et al.*, 1994). Both asbestos fibres and cigarette smoke then act as promoters to stimulate clonal proliferation of initiated cells. Pre-neoplastic epithelial cells in the lungs show histological evidence of altered differentiation (metaplasia) and altered proliferation (hyperplasia and dysplasia). Asbestos fibres have been shown to (i) induce metaplasia in tracheobronchial epithelium (Mossman *et al.*, 1977) and (ii) alter the temporal expression of proto-oncogenes that regulate proliferation of bronchial epithelial cells *in vitro* (Heintz *et al.*, 1993). Later stages in tumour development are characterized by additional genetic and cellular alterations leading to a locally invasive neoplasm followed by metastasis. The role of asbestos fibres in these later stages of tumour progression is unknown.

The proposed steps leading to the development of malignant mesothelioma are more speculative. Asbestos fibres may directly or indirectly injure and/or stimulate the proliferation of mesothelial cells. Fibres may be genotoxic to these proliferating cells. Alternatively, chronic stimulation of mesothelial cell proliferation may lead to the accumulation of spontaneous mutations that confer a proliferative advantage to pre-neoplastic cell populations. Fibres that persist in the interstitium or in the submesothelial connective tissue may trigger the chronic release of cytokines and growth factors from activated macrophages and thus lead to persistent stimulation of mesothelial cell growth. As in bronchogenic carcinoma, the role of asbestos fibres in the development of invasive and metastatic malignant mesothelioma is unknown.

Validation of mechanistic data in people exposed to asbestos or man-made fibres

The above proposed mechanistic hypotheses are based on experimental data derived from *in-vitro* models or *in-vivo* exposure of laboratory animals to

asbestos fibres. The endpoints of the in-vivo models have been validated by the production of the same spectrum of diseases observed in humans exposed to asbestos fibres, i.e. fibrosis, lung cancer and malignant mesothelioma (McClellan & Hesterberg, 1994). To use these mechanistic data in future evaluations of the carcinogenicity of natural and man-made fibres for humans, it is necessary to evaluate whether the same mechanisms are responsible for asbestos-related malignant diseases in both laboratory animals and humans. The following limited number of human studies have begun to investigate this issue:

Biomarkers in peripheral blood. Peripheral blood lymphocytes have been used as surrogates for lung cells to demonstrate an increased incidence of sister chromatid exchange in asbestos workers (Rom *et al.*, 1983). Workers exposed to quartz or asbestos fibres were also found to have increased levels of plasma lipid peroxides (Kamal *et al.*, 1989).

Bronchoalveolar lavage. Sampling of the bronchoalveolar compartment of the lungs in asbestos workers has confirmed persistent inflammation and the elevated release of ROS, cytokines, and growth factors from alveolar macrophages (Robinson *et al.*, 1986; Rom *et al.*, 1987; Zhang *et al.*, 1993). These changes are not entirely specific to exposure to asbestos fibres – they are also associated with other pneumoconioses and idiopathic pulmonary fibrosis (reviewed by Rom *et al.*, 1991).

Human lung and pleural biopsies. Biopsy or autopsy specimens of human lung and pleural tissues are an important resource with which to explore the cellular and molecular events in hyperplastic, dysplastic and neoplastic lesions associated with exposure to asbestos fibres. As reviewed by Gerwin (1994), this approach complements and confirms studies using cell lines derived from human bronchogenic carcinomas and malignant mesotheliomas. Few studies have been reported so far in patients with a history of asbestos exposure; additional studies are required to demonstrate specific cellular and molecular alterations in asbestos-related neoplasms.

Investigations using human tissue samples are essential to confirm whether the mechanisms identified in animal models also operate in humans. However, these studies must be designed carefully

and evaluated critically. Direct sampling of pre-neoplastic target cell populations is an invasive procedure; however, selection of surrogate samples such as bronchoalveolar lavage fluid, pleural effusion or peripheral blood must be validated. Appropriate control populations must also be evaluated in order to assess the specificity of a potential biomarker for asbestos exposure and to control for confounding factors such as cigarette smoking. The long latent period between exposure to fibres and the onset of symptoms of disease complicates evaluation of causal relationships. Despite these potential problems and limitations, additional cellular and molecular investigations of asbestos-related diseases using human tissues are essential. Workers exposed to man-made fibres who develop pleural plaques or effusions should also be included in these investigations (McClellan *et al.*, 1992).

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